Phycobilisome linker proteins are phosphorylated in Synechocystis sp. PCC 6803

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PHOSPHORYLATION OF PHYCOBILISOME LINKER PROTEINS IN *SYNECHOCYSTIS* SP. PCC 6803

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The controversial issue of protein phosphorylation from the photosynthetic apparatus of *Synechocystis* sp. PCC 6803 has been reinvestigated using new detection tools that include various immunological and *in vivo* labelling approaches. The set of phosphoproteins detected with these methods include ferredoxin-NADPH reductase and the linker proteins of the phycobilisome antenna. Using mutants that lack specific set of linker proteins and are affected in phycobilisome assembly, we show that the phosphoproteins from the phycobilisomes correspond to the membrane-, rod- and rod-core linkers. These proteins are in a phosphorylated state within the assembled phycobilisomes. Their dephosphorylation requires partial disassembly of the phycobilisomes and further contributes to their complete disassembly *in vitro*. *In vivo* we observed linker dephosphorylation upon *long-term* acclimation to higher light intensities, in conditions that lead to a turnover and a remodelling of phycobilisomes. Since the state of phosphorylation of linker proteins is not modified during *short-term* light acclimation we conclude that this phosphorylation process is not involved in State I/State II transitions. Rather, it is instrumental in the regulation of assembly/disassembly of phycobilisomes and may participate in signalling for their proteolytic cleavage and degradation.

Key words: phycobilisome linker proteins; phosphorylation/dephosphorylation; light-acclimation

INTRODUCTION

Phycobilisomes (PBSs) are large multimeric protein structures that function as an extrinsic light-harvesting antenna in cyanobacteria and red algae. They are located at the outer surface of thylakoid membranes where they transfer their excitation energy to the photosynthetic reaction centres that are embedded within the lipid bilayer. Our knowledge generated over more than 30 years from biochemical and biophysical analysis, genetics, electron microscopy and X-ray crystallography led to the improvement of the PBS structural model (1). In *Synechocystis* sp. PCC 6803 phycobilisomes are comprised of bilin-containing proteins, allophycocyanin (APC) and phycocyanin (PC), and colourless linker proteins that assemble PC and APC substructures and tune their properties in order to optimise energy transfer (2). Linker proteins can be divided into four groups: rod core linkers (L_{RC}^{28}) that attach the peripheral rods to the PBS core; rod linkers (L_R^{10}, L_R^{33}, L_R^{35}) that associate PC substructures into rod segments; the small core linker (L_C^{8}) that are associated with trimeric allophycocyanin at the peripheries of the core cylinders, and the core-membrane linker (L_{CM}^{99}) that acts in the organisation of the PBS core, in the PBS attachement to the membrane and also as the major terminal energy emitter to photosystem II (PSII; 1, 2, 3).

PBSs represent a major biosynthetic commitment of a cyanobacterial cell, they are also recruited as a principal nutrient source under starvation conditions besides their light harvesting function in photosynthesis. This dual function implies specific regulation for assembling, disassembling and remodelling the PBS structure according to changes in metabolic and energy requirements. Cyanobacteria have evolved several molecular mechanisms for acclimation of the PBS antenna that operate at the transcriptional (4, 5), translational and post-translational levels (6, 7). Taken together, these regulatory mechanisms contribute to remodelling PBS composition, size and number per thylakoid membrane when cells are exposed to environmental changes.

It has been suggested that chaperones may be essential in the first steps of PBS assembly when the degradation of biliproteins competes with protein biosynthesis (8). The subsequent modulation of PBS structures upon acclimation to various stresses leads in some cases to partial or full disassembly, degradation and reutilisation of phycobiliproteins. For instance, high rates of PBS degradation were observed for various cyanobacterial strains upon nutrient deprivation, and high light stress (6, 9-11). The *nbl* gene family encodes a major group of proteins that is involved in stress signalling and in controling PBS degradation. Two signalling components, a response-regulator and a histidine kinase, are encoded by the *nblR* and *nblS* genes, respectively (12,13), while two other genes code for NblA and NblB that are required for coordination of PBS degradation under nutrient deprivation (14-17). The cleavage of linker proteins has been suggested to be a prerequisite for complete degradation of phycobiliproteins (7, 18-20). Since PBSs

represent tightly organised structures it is not clear yet which protein determinants can trigger the initiation of the disassembly process and how such structural components as linker proteins, which are embedded in rod discs (21-23), can become accessible to regulatory proteins and proteolytic enzymes.

The facility by which phosphate groups can be added to a variety of amino acids within proteins and thereby induce changes in enzymatic activity, stability or binding properties, makes protein phosphorylation an attractive way of regulating cellular responses to the environment (24). In plant and algal chloroplasts, the light-harvesting antenna is a flexible structure that can accommodate changes in environmental conditions. Its post-translational modification by phosphorylation at some threonine residues that triggers changes in protein conformation and redistribution of the antenna between the two photosystems, in a process known as State transitions (25,26). Changes in protein phosphorylation also control the rate of proteolysis of the antenna proteins under excess illumination (27). Phosphorylation changes have been suggested to contribute to some mechanisms for PBS modifications a decade ago, but the experimental support to this view remained controversial (28). Most attempts to detect subunit phosphorylation within the PBS antenna in cyanobacteria were unsuccessful with one exception in which phosphorylation of ß-phycocyanin in *Synechococcus* sp. PCC 6301 was reported (29).

In the present study, we show that some linker proteins are permanently phosphorylated within the assembled phycobilisomes in *Synechocystis* sp. PCC. 6803. Their phosphorylation can be detected serologically with phosphospecific antisera as well as by the use of a fluorescence dye that recognises phospho-residues. We demonstrate that mutant strains that lack specific sets of linker proteins also loose these phosphoprotein signals. Dephosphorylation of linkers accompanies partial PBS disassembly and eventually leads to their total disassembly, a step that may be a prerequisite for protein degradation. Based on these data we discuss the physiological significance of linker phosphorylation in the response to changes in irradiance levels.

MATERIALS AND METHODS

Strains and growth conditions - *Synechocystis* sp. PCC 6803 and its derivatives were cultivated in BG11 medium (30) under white light of 50 µE m^{-2} s^{-1} at 30°C. For phosphorylation studies wild-type was grown at low light (LL; 50 µE m^{-2} s^{-1}) up to OD_{725} = 0.4 and then transferred to moderate (ML; 150 µE m^{-2} s^{-1}) or high light (HL; 400 µE m^{-2} s^{-1}) regimes for 36 h.

Extraction of thylakoid membrane and phycobilisome proteins - Thylakoid membrane proteins were isolated as described in (31). PBS were isolated as previously described in (18) with some modifications (7). The PBS preparation was based on cell disruption with glass beads, solubilization by 2% (v/v) Triton X-100 and subsequent separation of PBS by

0.25 – 0.79 M sucrose density gradient ultracentrifugation using 0.9 M phosphate buffer, pH 7.0. The fraction of intact PBS that formed the lower band in the gradient, was diluted in 0.9 M phosphate buffer, and centrifuged at 80.000 x g for 4 h at 4°C. The PBS pellet was dissolved in H_2O and frozen at –70°C.

Protein gel-electrophoresis, protein staining and immunological analysis - Thylakoid protein (3 to 5 µg chlorophyll per lane) and phycobilisome proteins were separated by 12.5% SDS-PAGE according to (32). For PBS loading, the absorbance of the samples was measured at 620 nm and an amount of biliproteins equivalent to OD_{620} = 1.5 was loaded per well. For protein visualisation, gels were stained with Coomassie Brilliant Blue, silver or luminescent dye SYPRO® Ruby (Molecular Probes, Leiden, Netherlands). Phosphoproteins were detected either by immunological analysis with phospho-threonine/serine antisera (Zymed, San Francisco) or staining with phosphoprotein gel dye Pro-Q™ Diamond (Molecular Probes, Leiden, Netherlands). For blocking unspecific reactions of phospho-threonine antisera, the inhibitors blocking phospho-serine residues (Zymed, San Francisco) were incubated with PVDF membranes prior to the immunological reaction. Antisera against PBS proteins were kindly provided by A. Grossman. The SYPRO® Ruby and Pro-Q™ Diamond stains were detected by a fluorescent image analyser FLA-300 (Fujifilm). Gels stained with SYPRO® Ruby and Pro-Q™ Diamond were scanned by orange filter O580 with excitation/emission wavelengths at 473/580 nm. For detection of PBS bilin-containing proteins by self-fluorescence, non-stained gels were scanned by red filter with excitation and emission at 635/675nm.

Phosphorylation of cyanobacterial proteins in vivo - The phosphorylation of *Synechocystis* cellular proteins *in vivo* was performed according to (33). Cells were grown in BG11 medium under white light of low intensity (LL; 50 µE m^{-2} s^{-1}) to an OD_{725} of 0.6. Cells were harvested by centrifugation and resuspended in BG11 medium without phosphate to an OD_{725} of 3. For each sample, 1 ml of cell culture was transferred to a small Petri dish to provide equal illumination. The phosphorylation was performed by addition of 30 µCi of carrier-free [$^{32}P_i$] with subsequent incubation under LL for 1.5 h. After incubation cyanobacterial proteins were precipitated with 5% (w/v) trichloracetic acid (TCA), washed with 100% acetone and dissolved in SDS sample buffer for Laemmli-gel system.

Dephosphorylation by alkaline phosphatase and proteolysis in vitro - For dephosphorylation of thylakoid and PBS proteins, samples were incubated with bovine alkaline phosphatase (SIGMA, Schnelldorf) according to (34). Phycobilisome proteins were incubated with alkaline phosphatase in a buffer containing 0.1 M glycine, pH 10.4, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ for 30 min at room temperature. The reaction was stopped by addition of 20 mM EDTA.

RESULTS

Phosphorylation of thylakoid proteins in the wild-type Synechocystis *strain* - Phosphorylation of photosynthetic proteins in *Synechocystis* was analysed by two assays, immunological detection with antisera raised against phosphorylated amino acid residues or by *in vivo* phosphorylation with carrier-free [^{32}Pi]. For immunological analyses thylakoid-enriched protein fractions were extracted from cyanobacterial cells grown under standard conditions. After SDS-PAGE protein profiles were visualised by silver-staining (Fig. 1A, lane 1) and transferred onto PVDF membrane for immunological detection of phosphorylated proteins (Fig. 1A, lane 2). The application of phospho-threonine and -serine antisera resulted in similar phosphoprotein profiles (data not shown). To check whether similar phosphoprotein patterns resulted from serological crossreactivity of phospho-threonine and -serine residues or from the presence of both phospho-residues, inhibitors blocking phospho-threonine or -serine antisera were applied during the immunological assay. Since these treatments did not change significantly the profile of phosphorylated proteins (results not shown) we assumed that phosphorylation occurred at both, threonine and serine residues. In subsequent studies we restricted analysis to the use of phospho-threonine antisera. Between 10 and 15 phosphoproteins were detected in thylakoid preparations with antisera elicited against phospho-threonine residues (Fig. 1A). The strongest phosphorylation signals were obtained for proteins with molecular masses of 16, 20, 23, 29, 33, 35, 40, 50 and 90 kDa. Obviously these proteins represent a set of phosphoproteins that are stable enough to be detected by an immunological approach. In order to detect phosphoproteins that undergo rapid phosphate exchanges we employed an *in vivo* labelling approach with [^{32}Pi]. This experiment identified another set of phosphoproteins in thylakoid membranes (Fig. 1A) predominantly in the ranges of 18 – 20, 30 - 36 kDa and a highly phosphorylated band of 66 kDa. Comparison of silver-stained thylakoid membrane polypeptides with those of the immunological analysis and [^{32}Pi] labelling suggested that the major phosphorylated proteins migrated in an electrophoretic position of phycobilisome subunits. Since the phosphorylation of the PBS antenna is a controversial issue more than a decade, we applied the same immunological assay to a purified PBS fraction. PBS antenna proteins were extracted from *Synechocystis* cells on sucrose gradients and separated by SDS-PAGE. Proteins were then visualised by staining with silver nitrate or fluorescence dye SYPRO Ruby (Fig. 1B). The preparation of PBSs contain predominantly two groups of proteins, heavily stained phycobilin-containing proteins in the molecular weight range of 16 – 22 kDa, and less abundant proteins corresponding to the FNR protein and non-bilin-containing linker peptides, rod linkers L_R^{33} and L_R^{35}, rod-core linkers L_{RC}, as well as to mature and truncated forms of membrane linker L_{CM}^{99} and L_{CM}^{76}, respectively (18)

SYPRO Ruby, which is a quantitative and highly sensitive dye for protein detections stained saturating amounts of phycobiliproteins (APC and PC) negatively. Phosphorylated proteins were visualised with phospho-threonine antisera or the fluorescence dye Pro-Q Diamond that reacts with phosphorylated residues (Fig. 1B). Both assays revealed five heavily phosphorylated bands that corresponded to linker proteins (L_{CM}^{99}, L_R^{35}, L_R^{33} and L_{RC}) and FNR which were also markedly labelled in thylakoid membrane preparations assayed with phosphospecific serum. Note on Fig. 1B a limited shift in position of the FNR between lanes „Pro-Q" and „Autofluo" versus lanes „Silver" and „SYPRO Ruby" due to changes in gel sizes during various staining and blotting procedures. Although two highly phosphorylated bands in the molecular weight range between 16 and 22 kDa were also detected in thylakoid membrane preparations (Fig. 1A, lane „Phospho-thr" and „^{32}Pi"), no signals in the same region could be visualised in PBS at least with the phospho-threonine antisera (Fig. 1B). Comparable results were obtained with the fluorescent Pro-Q Diamond dye although it displayed additional signals in the range of PC/APC. To understand the nature of the signals originating in PC/APC proteins the non-stained gel loaded with PBS proteins was scanned with a red laser set at an excitation/emission wavelength suitable for detection bilin-containing proteins. (Fig. 1B, lane „Autofluo"). We observed a very weak signal comigrating with FNR and intense signals from the PC and APC bands. This showed that the PC/APC signals obtained from Pro-Q staining corresponded to self-fluorescence of bilin proteins and not to phosphorylation of PC/APC.

Phosphorylation of thylakoid proteins in PBS-deficient mutants - In order to assess whether the phosphorylation signals actually originated from the PBS linkers rather than from minor comigrating contaminating minor polypeptides, the immunological assay was applied to mutant strains that either had a decrease in PBS sizes because of a lack of rods (CK mutant) or that fully lacked PBS antenna (PAL mutant). The PAL mutant bears a deletion in genes encoding the L_{CM}^{99} and allophycocyanin proteins in a phycocyanin deficient background (35). The CK mutant is deleted in the entire *cpc*- operon (Ughy and Ajlani, unpublished). PBSs were still present in CK but contained only APC and were smaller in size (Fig. 2A). Both strains were analysed for the presence of all linker proteins by immunodetection with antisera raised against the various linker and bilin-containing polypeptides (Fig. 2B). According to immunological analysis none of the PBS proteins was present in the PAL strain. In the CK strain the absence of phycocyanin rods and rod linkers $L_R^{33, 35}$ prevented the accumulation of newly synthesised L_{RC} which connect the APC-containing core and the PC rods (Fig. 2B). These rod core linkers were still detectable in the CK strain but to a much lower extent than in the wild-type. The CK strain still contained allophycocyanin in PBS core and L_{CM}^{99} linker in a similar amount as in the wild-type. The phosphorylation pattern in thylakoid

membranes of wild-type, PAL and CK mutant strains was also probed with phospho-threonine antisera (Fig. 2C). PBSs extracted from the wild-type were loaded as a control for migration of linker proteins. The differences in the intensity of protein phosphorylation profile of wild-type thylakoid membranes as compared to the one presented in Fig. 1A resulted from a weaker exposure of ECL-labelled films in order to visualise a better contrast with the protein patterns in the PBS-deficient mutants. High resolution of the 29 – 36 kDa region revealed three phosphorylated bands in the range of rod linkers with the central one showing no correspondence to PBS subunits. No phosphorylated bands were detected at any linker position in the PAL mutant (Fig. 2C). The core-membrane linker remained detectable as a phosphoprotein in the CK mutant at the same rate as in the wild-type, while no phosphorylated band remained at the positions of the other linkers. Since the absence of linker polypeptides in the mutants resulted in the loss of the corresponding phospho-threonine signals, we concluded that the rod linkers were phosphorylated in the phycobilisomes of the wild-type.

Dephosphorylation occurs only in partially disassembled phycobilisomes - As an additional proof of linker phosphorylation *in vivo*, PBSs were dephosphorylated *in vitro* by incubation with alkaline phosphatase that is specific for O-phosphorylation of threonine or serine residues. We observed only partial dephosphorylation of the linker proteins (Fig. 3A). Since linker proteins are embedded in PC hexamers, we reasoned that the dephosphorylation sites might not be easily accessible to the phosphatase in the assembled PBSs. When isolated PBSs are resuspended in water or in phosphate buffer of very low concentration, they are partially disorganized (36). To check whether dephosphorylation of linker proteins is controlled by the assembly state of the PBSs, we run the pre-treated or non-treated PBS preparations with alkaline phosphatase in sucrose gradients. As shown by the silver-staining pattern of the 16 – 22 kDa region containing the PC/APC proteins, this experiment separated intact PBSs found at the bottom of the gradient (fractions 1 in Fig. 3B), from partially or fully disassembled PBS subunits found at the top of the gradient (fractions 2 to 20 in Fig. 3B). Phosphorylated linker proteins in the region of 24 – 37 kDa were detected immunologically with an anti-phospho-threonine serum and are presented in the lower panels of Fig. 3B. Without alkaline phosphatase treatment, linker proteins were heavily phosphorylated in fraction 1 but less efficiently in fractions 9 – 16 where most PBS proteins were detected by silver nitrate staining. After dephosphorylation by alkaline phosphatase, the PBS preparation resolved phosphorylated linkers only in intact PBSs (fraction 1), but no other phosphorylation was observed all over the gradient fractions (lower panel, + AP). At the same time the phycobiliproteins were all shifted to the upper part of the gradient indicating that they underwent further disassembly. This experiment demonstrates that linker proteins are subjected to dephosphorylation only in partially disassembled PBS structures and that

their dephosphorylation enhances disassembly of the PBS antenna.

Dephosphorylation of PBS linkers is enhanced under high light acclimation - Light is one of the major environmental factors which is involved in the modification of light-harvesting antenna in higher plants and cyanobacteria.

Phosphorylation/dephosphorylation of light-harvesting antennae in higher plant chloroplasts, which is involved in state transitions and in antenna degradation, is controlled by light intensity and quality. The effect of light on the phosphorylation of linker proteins was analysed in wild-type *Synechocystis* that was acclimated to moderate (ML) and high light (HL) for 36 h. The phosphorylation rate of thylakoid membrane proteins was detected immunologically (Fig. 4A). A comparative analysis of phosphoproteins under various light regimes demonstrated some changes in the phosphorylation rate of a few proteins including PBS-linker proteins (Fig. 4A). Since the acclimation of photosynthetic membranes to higher light regimes also causes a reduction in amount of PBS linker proteins which is controlled at the transcriptional, translational and posttranslational levels (7), the ratio between protein dephosphorylation and protein amount was analysed with specific antibodies against L_{CM}^{99}, L_R^{35} and L_R^{33} (Fig. 4B). Western analysis demonstrated that L_{CM}^{99} and L_R^{33} amounts decreased under HL but to a much lower extent than their corresponding dephosphorylation. The amount of L_R^{35} linker remained unchanged whereas is was heavily dephosphorylated under high light. The phosphorylation/dephosphorylation rate of linker peptides was not changed during short-time acclimation corresponding to state transitions (data not shown).

DISCUSSION

Various *in vivo* and *in vitro* labelling studies have indicated phosphorylation of thylakoid membrane proteins in various photosynthetic prokaryotic organisms (summarized in 37), like *Calothrix* sp. PCC 7601 (38), *Synechococcus* sp. PCC 6301 (39, 40), *Synechococcus* sp. PCC 7942 (41) and *Synechocystis* sp. PCC 6803 (42). The labelling of proteins in *Calothrix* sp. PCC 7601 revealed three phosphoproteins in thylakoid membranes phosphorylated on serine and threonine residues (38). In *Synechococcus* sp. PCC 6301 labelling experiments *in vivo* with [^{32}Pi] orthophosphate demonstrated phosphorylation of an 18.5 kDa protein which was also found in a purified PBS fraction and has been proposed to represent ß-phycocyanin (43, 44). Since these early observations of protein phosphorylation in thylakoid membranes only one additional protein, the PsbH component of photosystem II was claimed to be phosphorylated in cyanobacteria (45) although conflicting observations were reported ten years later (46).

In the present study, the phosphoproteins of *Synechocystis* thylakoid membranes were analysed by three strategies. The first ones are based on an immunological detection with antisera raised against phosphorylated amino acid residues and on the

application of a fluorescent dye recognising phosphate groups attached to tyrosine, serine and threonine residues. The third one consists in an *in vivo* incubation of *Synechocystis* cells with radiolabelled orthophosphate. Approximately 15 phosphoproteins could be detected using these approaches in the thylakoid membranes of *Synechocystis*. The profiles of phosphoproteins detected by these procedures were qualitatively and quantitatively different. Phosphospecific antisera and the fluorescent dye detect proteins which carry phosphorylated amino acid groups that may be very long-lived, while radiolabelling with [^{32}Pi] identifies those proteins which are post-translationally modified during the incubation time, in particular those residues that are reversibly phosphorylated.

The most easily detectable and highly phosphorylated proteins turned out to be FNR and linker proteins (L_{CM}^{95}, L_R^{35}, L_R^{33} and L_{RC}). The phosphoprotein with a molecular weight of about 30 kDa previously detected with a phosphothreonine antisera (47), could correspond to one of the rod linker proteins. We did not observe any phosphorylation of the major bilin-containing antenna proteins, allophycocyanin and phycocyanin, which contrasts with a previously published report (29). The FNR, which was shown to be associated to PBSs (48) was substantially phosphorylated in our antenna preparations. The phosphorylation of FNR from higher plant chloroplasts at serine and threonine residues has been observed, but the function of this process remains poorly understood (49). The profile of phosphorylated proteins in wild-type and cyanobacterial mutant strains deficient in phycobilisomes (PAL strain) or only rod linker proteins (CK strain) differed in these bands corresponding to the missing linkers in the mutants. This proved that the signals with phospho-threonine antisera corresponded to linker proteins.

The detection of substantial signals of linker proteins with anti-phospho-threonine sera contrasted with their poor phosphorylation by orthophosphate [^{32}Pi]. This indicated that linker phosphorylation at serine or/and threonine residues is quite a stable post-translational modification. Surprisingly, the rod linkers were poorly sensitive to a dephosphorylation treatment and their dephosphorylation in PBS preparations by alkaline phosphatase did not exceed 50%. This could be explained by the localization of linker proteins, connecting phycocyanin segments in the internal cavities of the disks (22, 23). Therefore, externally added enzymes have little access to the linkers within the fully assembled PBS structures. The separation of partially disassembled PBS that were pre-treated with alkaline phosphatase demonstrated that indeed only partially or completely dissociated PBSs can be fully dephosphorylated while linkers in fully assembled PBSs are not accessible. Moreover, we observed that dephosphorylation of linkers in partially assembled PBSs further disassembled their constitutive subunits. This experiment suggests that dephosphorylation of linkers could participate in the mechanism governing the remodeling and turnover of the

PBSs in *Synechocystis*. Indeed, we observed that the state of linker protein phosphorylation changed with the light regime for growth. The acclimation of cells for at least 24 h to high light resulted in decreased phosphorylation rate of linker proteins. It is well known that upon acclimation from low to high light, PBS antenna can be down-sized due to modulation of expression levels of genes encoding phycobiliproteins (4, 5) and through PBS degradation (6, 7). The latter process involves the protease SppA1 that causes the cleavage of linker proteins with subsequent release of distal phycocyanin rod segments (7). Besides an expected drop in amount of phycobilisomes upon acclimation to higher light, the dephosphorylation rate was more pronounced than the reduction in the amount of PBS proteins. This observation argues for a role of linker dephosphorylation *in vivo* in PBS remodeling.

Linker dephosphorylation may act as a signal for protein degradation once PBS disassembly has started, while protein phosphorylation could occur before or during assembly of PBS hexamers. The initial steps of biliprotein biosynthesis include competition between protein biosynthesis and degradation. The biliprotein subunits with no attached bilins or those lacking partners can be rapidly subjected to degradation (summarised in 8). The mechanisms controlling biliprotein degradation are unknown although one of the suggested models is the binding of chaperone proteins which would activate a degradation pathway (8). In that case phosphorylation may play an important role in the stabilisation of linker polypeptides in the PBS assembly pathway, while their unphosphorylated state could be signalling for their degradation. Changes in protein phosphorylation modify folding that in turn change their affinity for proteolytic enzymes and assembly partners. The role of linker (de)phosphorylation in cyanobacteria would then be similar to that of the phosphorylation of LHCII and D1 protein in higher plant chloroplasts that are considered to be targeted to degradation upon dephosphorylation (27, 50). A long phase of acclimation to higher light intensities of at least 24 h is required for the detection of PBS protein dephosphorylation. Such a lag period in the acclimation was also observed for light-harvesting complex of higher plants (27). This argues for an activation of some substrates or/and enzymes involved in the protein dephosphorylation and/or cleavage.

Up to now it is not clear how PBS structures can be loosen during nutrient deprivation or light stress, conditions which cause a massive degradation of cyanobacterial antenna (10, 51, 52). The major protein family which is known to be involved in the degradation of PBS is encoded by *nbl* gene family. One of the components, NblA, is directly involved in PBS degradation under nutrient deprivation (14, 16, 17). It is suggested that binding of NblA to phycocyanin can mark proteins for their further recognition by proteases or can soften the PBS structure by increasing the distances between single phycocyanin segments. It is then expected that proteases and phosphatases will get higher access to their substrates. These and other

questions could be approached by studies on NblA and cyanobacterial phosphatases.

FOOTNOTES

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The abbreviations used are: APC, allophycocyanin; HL, high light; LL, low light, ML, moderate light; PBS, phycobilisome; PC, phycocyanin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

FIGURE LEGENDS

Fig. 1. Detection of phosphosubstrates in isolated thylakoids (A) and purified PBS antenna (B) of *Synechocystis* wild-type strain. Thylakoid and phycobilisome proteins were isolated from wild-type *Synechocystis* cells grown under standard light regime and separated by 12.5% SDS-PAGE. Protein profiles were visualised by silver-staining (A and B) or by fluorescence dye SYPRO Ruby (B). Protein phosphorylation was detected for thylakoid membrane and PBS proteins with phospho-threonine antisera (A and B), fluorescent dye Pro-Q Diamond (B,) or by *in vivo* labelling (A). The fluorescence of bilin-containing proteins (PBP), APC and PC, was detected by scanning the non-stained gels with a red laser as described in M&M (B; lane „Autofluo"). Protein molecular weight markers are indicated at the left and right of panels A and B, respectively; PBS proteins are marked at the left of the panel B (L_{CM}^{99}, membrane linker; $L_R^{33,\ 35}$, rod linkers, L_{RC}, rod core linkers). Phosphorylated protein bands in Panel A comigrating with PBS proteins are marked by arrows.

Fig. 2. Analysis of phosphoproteins in the wild-type and mutant strains deficient in PBS proteins. (A) PBS antenna extracted from the wild-type and linker-deficient CK strain. PBS proteins separated by SDS-PAGE were stained with Coomassie Brilliant Blue. (B) Western analysis of PBS proteins in thylakoid membranes of the wild-type, PAL and CK strains. Thylakoid proteins from wild-type, PAL, and CK strains were separated by electrophoresis in a 12.5% SDS-PAG and proteins were probed with antisera raised against various core membrane, rod, rod-core linkers, and APC/PC (indicated at the left). (C) Phosphoproteins in thylakoid membranes of the wild-type and PBS deficient strains. Phosphorylated proteins were identified by immunodetection with phospho-threonine antisera. PBS extracted from wild-type cells were used as a control to identify linker proteins among other phosphorylated thylakoid proteins.

Fig. 3. Dephosphorylation of PBS linker proteins by alkaline phosphatase and their separation in sucrose gradients. (A) Isolated PBS were incubated in the absence or presence of alkaline phosphatase (-AP and +AP) and separated on 12% SDS-PAGE. The degree of phosphorylation was immunologically detected with anti-phosphothreonine serum. (B) PBS fractions treated and non-treated with alkaline phosphatase were separated on 0.25 – 0.79 M sucrose gradients. Fractions of 0.2 ml were collected from the bottom of the gradient and separated by 12% SDS-PAGE. Proteins were detected by silver-staining (the upper panels of the experiments with and without AP) and by immunological analysis with phospho-threonine antiserum (lower panels). From silver-staining, only the 16 – 22 kDa region with APC/PC proteins (PBP) is presented. For immunological analysis, the part of the gel of 24 – 38 kDa with rod and rod core linkers is shown.

Fig. 4. Phosphorylation levels of thylakoid proteins upon acclimation to various light regimes. *Synechocystis* wild-type cells were grown under LL and then acclimated to ML and HL for 36 h. Thylakoid proteins were separated by 12.5% SDS-PAGE and the phosphorylation rate was detected by immunological analysis with phospho-threonine antisera (A). The amount of linker proteins was controlled by antisera against linker proteins (B). Arrows indicate unknown phosphoproteins with a light-dependent phosphorylation rate.

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